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Article

Complex pectin metabolism by *Lactobacillus* and *Streptococcus* suggests an effective control approach for Maillard harmful products in brown fermented milk

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ABSTRACT

Harmful Maillard reaction products (HMRPs) derived from brown fermented milk pose a potential threat to human health, but the conversion mechanism during the manufacturing process remains elusive and urgently needs to be controlled. Acrylamide (FC 2.14, adjusted *p*-value = 0.041), 5-hydroxymethylfurfural (FC 2.61, adjusted *p*-value = 0.026) and methylglyoxal (FC 2.07, adjusted *p*-value = 0.019) were identified as the significantly increased HMRPs after browning in this study and the analysis of proteomics integrated with untargeted metabolomics demonstrated that the degradation of HMRPs was jointly accomplished by *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The galactose oligosaccharide metabolism in *Streptococcus thermophilus* was identified as a key biochemical reaction for HMRPs degradation, and the hydrolysates of pectin could be utilized as prebiotics for *Streptococcus thermophilus*. Eighteen classes of enzymes of *L. bulgaricus* and *Streptococcus thermophilus* related to energy metabolism were upregulated in the pectin-added group, indicating that the entry of acrylamide and methylglyoxal into the tricarboxylic acid cycle was accelerated. NAD-aldehyde dehydrogenase and alanine dehydrogenase are enzymes belonging to *Streptococcus thermophilus,* and their downregulation accelerated the efflux of acetate, which was beneficial for the proliferation of *L. bulgaricus* and prevented the conversion of pyruvate to L-alanine, thus facilitating the energy metabolism. The recoveries and relative standard deviations of the intraday and interday precision experiments were 89.1%-112.5%, 1.3%-8.4% and 2.1%-9.4%, respectively, indicating that the developed approach was credible. Sensory evaluation results revealed that the brown fermented milk added with pectin had a better flavor, which was due to the fact that the supplement of polysaccharide promoted the fatty acid metabolism of lactic acid bacteria and increased the aroma substances including octoic acid and valeric acid. This study provided an insight into the formation and degradation mechanism of HMRPs in brown fermented milk, aiming to reduce the intake of advanced glycation end products in the diet.

1. Introduction

Brown fermented milk is popular for its nutritional benefits and appealing flavor in Europe, China, Japan and Southeast Asia. In contrast to traditional fermented dairy products, brown fermented milk is produced by incubating milk for an extended period at a high temperature first to allow the Maillard reaction between reducing sugar and proteins and then which was fermented by *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. It had been indicated that the concentrations of organic acids, especially medium chain fatty acids, and peptides in brown fermented milk increased significantly compared with those in conventional fermented milk, which contributed to the balance between sour and sweet taste thereby greatly improving the sensory quality and nutritional value of fermented dairy products [1]. However, previous research evidenced that thermal treatment can also induce potentially harmful Maillard reaction products (HMRPs), such as Nε-(carboxymethyl)lysine, acrylamide and 5-hydroxymethylfurfural (5-HMF), which had been reported to be associated with various inflammatory conditions and possibly cause chronic diseases, including diabetes, kidney disease, and Alzheimer's disease [2]. Therefore, it is urgent to take effective measures to control the formation of harmful substances and investigate the degradation mechanism of HMRPs during the production of brown fermented milk so as to reduce the exposure risk.

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Hydrocolloids have been considered as one of the most effective approaches to mitigate the formation of toxic by-products in Maillard reaction. Pectin is a kind of hydrocolloid belonging to anionic polysaccharide, which has attracted much attention as a widely used natural food additive like stabilizer, prebiotics, emulsifier and thickener in fermented dairy products. Both of chemical model and real food systems demonstrated that pectin is a promising natural substance that can effectively reduce the formation of acrylamide. Passos et al. found that the inhibition rate of acrylamide generation reached up to 67% during the Maillard reaction when biscuits were prepared with 5% (*w/v*) pectin. The lowering effect of pectin on the surface tension of impregnated solution and the combination of pectin and divalent metal salts to form a coating on the surface of food is a possible mechanism of inhibition on the formation of acrylamide, while further studies are required to characterize the inhibitory mechanism of pectin on the formation of HMRPs [3]. Zeng et al. demonstrated that 2% (*w/v*) pectin solution can inhibit more than 50% acrylamide formation in the asparagine/glucose model while immersing potato strips in 5% (*w/v*) pectin solution for 1 hour can reduce the formation of acrylamide by 20% [4]. Another study also reported that 1% (*w/v*) pectin was effective (47%) in preventing the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine in chemical model system [5]. However, the preliminary study concerning the effect of pectin with different esterification degrees on acrylamide formation in the model system showed that only high-methoxyl pectin can significantly reduce the generation of acrylamide and inferred that the inhibition of pectin on acrylamide formation is conditional and selective [6]. In addition, these data also demonstrated that the behaviors of chemical agents could be profoundly different in chemical models and real food systems, therefore a real food investigation is necessary to corroborate the data from chemical model analysis.

To improve the quality and safety of brown fermented milk, Han et al. investigated the formation and alteration of HMRPs and proved that the browning stage was the period for the generation of HMRPs. During fermentation process, the concentrations of HMRPs were significantly reduced in brown fermented milk, suggesting that the regulation of microbial life activities was a probable potential approach for HMRPs degradation, while the underlying mechanisms remained unknown due to the complex matrix of brown fermented milk [7]. Previous studies have focused on the inhibition of pectin on Maillard reaction byproducts, ignoring the possibility of pectin in brown fermented milk as potential prebiotics to regulate the life activities of microorganisms and promote the degradation of harmful products of the Maillard reaction, which provided an idea for controlling the generation of HMRPs in brown fermented milk.

Omics research strives to comprehensively characterize and quantify pools of molecules in a complex matrix. Advances in high-through methods such as proteomics and metabolomics can provide insights into transcriptional regulation and metabolic flux [8]. Proteomics complements the ability of the biomics approach to unveil the biological profiles that may be missing based on metabolomics methods and help to identify the critical proteins responsible for these differences [9]. Metabolites are the end products of all regulation and can better represent cell phenotype than transcriptomics and proteomics [10]. Compared to single-omics, multi-omics approach can incrementally confirm the associations and interactions of features from genotypes to phenotypes, comprehensively demonstrating the complex biological system in living organisms. Jia et al. investigated the metabolic pathways and nutritional quality of goat milk contaminated with sodium perchlorate through untargeted metabolomic and proteomics methods. UHPLC-Q-Orbitrap HRMS was applied to unveil the differences between different sodium perchlorate concentrations and identify the pivotal metabolites [11].

In this study, a label-free shotgun proteomics approach combined with untargeted metabolomics based on UHPLC-Q-Orbitrap HRMS was established to elucidate the degradation mechanism of HMRPs in brown fermented milk during fermentation and to further explore the molecular mechanism of the effects of the addition of high-methoxyl pectin on the metabolic activities of *L. bulgaricus* and *Streptococcus thermophilus*. A mass spectrum information optimization program (MS-IOP) linked with chemometrics was applied to extract and analyze massive mass spectrometry information and screen significant differences highly related to HMRPs. The signature metabolites and proteins obtained were further investigated for pathway and network analysis to gain mechanistic insights into the biological system. The aim of this work was to provide a reliable strategy for controlling HMRPs formation in brown fermented milk without impairing sensory properties and open up more possibilities for the optimization of manufacturing conditions.

2. Materials and methods

2.1. Chemicals and regents

Skim milk powder (protein 33%) was obtained from Fonterra Co. Ltd., Auckland, New Zealand. Citrus pectin (150,000 g mol^{-1}) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Chromatography-grade reagents, including methanol, acetonitrile and acetonitrile were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (≥ 95%) and ammonium formate (≥ 99.9%) were from Aladdin Reagent Co., Ltd. (Shanghai, China). Ultrapure water was obtained by a Milli-Q Plus Water System (Bedford, MA, USA). All analytical standards (≥ 97%) used for quantification in this article were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland) and Mallinckrodt Baker (Phillipsburg, NJ).

2.2. Sample preparation

Forty brands of skim milk powder samples packed in metal pail containers were purchased from 15 different regions as biological replicates to minimize the sample-to-sample variations and normalize the effect of milk from different sources. Experimental units were prepared by dry-blending, and each brand of skim milk powder was mixed in equal proportion and homogenized via vigorous shaking in a Turbula® Shaker for 15 min. Set-type dairy products for the experiment were prepared as described by Sah et al. with some modifications [12]. Starter culture was used according to the manufacturer's instructions. The reconstituted milk was prepared by suspending 10% (*wt/wt*) skim milk powder in water and then homogenizing it (65 °C, 90 MPa) using a high-pressure homogenizer (Hirayamaha-300 M). Pectin was added to the skim milk solution at 0 g kg^{-1}, 0.05 g kg^{-1}, 0.1 g kg^{-1}, 0.15 g kg^{-1}, 0.2 g kg^{-1}, 0.25 g kg^{-1}, and 0.3 g kg^{-1}, denoted as the control, 0.5% pectin, 1% pectin, 1.5% pectin, 2% pectin, 2.5% pectin and 3% pectin groups, respectively. The mixture was sterilized at 95 °C and maintained for 3 h, followed by cooling to 42 °C. Then, *L. bulgaricus* and *Streptococcus thermophilus* (1×10^6 CFU mL^{-1}) were inoculated into the sterilized milk, and the mixture was fermented at 42 °C until the pH reached 4.5 (70 °T) according to the national food safety standard for fermented milk (GB 19,302–2010) and a rule entitled *Milk and Cream Products and Yogurt Products; Final Rule to Revoke the Standards for Lowfat Yogurt and Nonfat Yogurt and to Amend the Standard for Yogurt* issued by the Food and Drug Administration on June 11, 2021. Routine fermented milk was prepared in the same way as the brown fermented milk except that they were pasteurized at 95 °C for 15 min and cooled to 42 °C. Brown fermented milk with or without pectin and conventional fermented milk were collected before and after fermentation for analysis. Each group of samples was analysed in six replicates, and the samples of each experiment were analysed in quadruplicate as technical repeats ($n = 6 \times 4$). All samples were stored at −80 °C before analysis. Matrix-fortified calibration curve stands were prepared by adding the analytes into blank samples at a concentration range of 0.1–10,000.0 $\mu g\ L^{-1}$. Stock solutions of individual compounds were prepared in methanol (1 mg mL^{-1}) and stored at −20 °C in dark. Then, the working standard solutions were prepared by adding suitable aliquots of each individual standard stock solution (10 mg L^{-1}) and diluting them with appropriate

amounts of methanol and stored in screw-capped glass tubes at −20 °C in dark.

2.3. Metabolomics analysis

Metabolite extraction was conducted according to the validated protocol with minor modifications. Briefly, 1 mL of the sample was pipetted and mixed with 1 mL cold acetonitrile (80%, 4 °C) in a 5-mL eppendorf tube for 3 min and sonicated in an ice bath for 10 min. Then, the sample was centrifuged at 4 °C (10,000 × *g*) for 20 min and filtered through a 0.22-μm microporous membrane.

Quality control (QC) samples were prepared by combining equal portion of all samples and injected every 3 samples to evaluate the mass accuracy and ensure the stability of the system and conditions.

A Q-Exactive Orbitrap HRMS coupled to a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific, San Jose, USA) equipped with Hypersil GOLD column (C18, 100 × 2.1 mm, 5 μm) was applied to the collection of metabolite information. The column temperature was maintained at 35 °C and the parameters of injection volume and elution rate were 5 μL and 300 μL min^{-1}, respectively. Mobile phase A was water and eluent B was acetonitrile, both of which were added with 0.1% formic acid and 4 mM ammonium formate. The liner gradient elution condition was set as follows: 0–1 min, 20% B; 1–3 min, 20–65% B; 3–6 min, 65–85% B; 6–7 min, 85–100% B; 7–14 min, 100% B; 14–14.1 min, 100–20% B; 14.1–15 min, 20% B.

The heated-electrospray ionization source (HESI) was operated in both positive- and negative-ion mode under the following instrument parameters: auxiliary gas heater temperature, 320 °C; vaporizer temperature, 350 °C; spray voltage, ± 3.5 kV; sheath gas flow rate, 35 arbitrary units; auxiliary gas flow rate, 10 arbitrary units. The mass spectrometer was operated in a Full MS scan (*m/z* 50–1000) and acquired in Orbitrap with resolution of 70,000 FWHM followed by the data-dependent MS/MS (dd-MS2) acquisition mode. Activation type was high energy collision dissociation (HCD) with a spectra resolution as 35,000 FWHM and normalized collision energies were set as 17.5 eV, 35 eV and 52.5 eV, respectively.

2.4. Proteomics analysis

Parallel groups of samples were centrifuged at 10 °C (10,000 × *g*) for 30 min to remove lipids, and the skim milk samples were rinsed three times with 0.1 mol L^{-1} PBS (pH 6.8). Proteins were digested according to the modificatory method of the filter-aided sample preparation (FASP). The protein extract (100 μL) of each group was reduced with 10 μL of 10 mM dithiothreitol at 56 °C for 60 min, and then alkylated with 15 μL of iodoacetamide (55 mM) in dark at room temperature for 30 min to block reduced cysteine residues. The obtained samples were transferred to ultrafiltration centrifuge tube (10 kDa, Merck Millipore, Billericia, MA, USA) and centrifuged (10,000 × *g* for 15 min) three times with 100 μL UA buffer (8 M Urea, 150 mM Tris–HCl pH 8.0) and DS buffer (50 mM triethylammonium bicarbonate at pH 8.5), respectively. Finally, protein samples were digested with trypsin in 50 mM NH_4HCO_3 (trypsin: substrate = 1:50 (*w/w*)) at 37 °C and incubated for 10 h and the hydrolysis system was terminated by adding formic acid (1%). The filtrates were desalted by C_{18} cartridges (Millipore, corp, Bedford, MA, USA) and collected with Oasis HLB cartridges (Waters Cooperation, USA).

The collected peptide mixture was then loaded onto a Hypersil GOLD column (C_{18}, 100 × 2.1 mm, 5 μm) and separated through linear gradient elution with mobile phase consisting of A (0.1% formic acid) and B (84% acetonitrile dissolved with 0.1% formic acid) at a flow rate of 0.3 mL min^{-1}: 95–65% A at 0–50 min, 65%–0% A at 50–75 min, 0%A at 75–90 min, 0%–95% A at 90–90.1 min, 95% A at 90.1–100 min. The mass spectra of protein were gathered by a Quadrupole-Orbitrap HRMS in Full MS/dd-MS2 (*m/z* 200–2000) acquisition workflow. Mass resolutions of full MS scan and ddMS2 were 70,000 FWHM and 17,500 FWHM, respectively.

2.5. Identification and quantification of metabolites

The MS-IOP data analysis process was performed according to the previous description with minor modification. The resulting raw data of metabolomic was exported from Xcalibur 4.0 software (Thermo Fisher Scientific) and processed through Compound Discover 3.2 (Thermo Fisher Scientific) to extract all chromatographic peaks for substances identification based on the following parameters: the signal-to-noise ratio of molecular and the minimum peak intensity were set as 3 and 1.0×10^6, respectively. A total of 3684,337 spectra of substances were obtained from 192 total ion chromatograms of metabolite samples. Identification of unknown compound was carried out after compensating differences in retention time (RT) and integrating exact mass and MS/MS fragments. By comparing adduct states including $[M+NH_4]^+$, $[M+H]^+$, $[M-H]^-$, $[M+HCOO]^-$ and MS2 data with databases, a feature table containing accurate mass (mass tolerance < 0.01 Da), RT (tolerance < 0.1 min), name and formula of metabolite was obtained. Citing the identification of D-3-deoxyglucosone as an example (Fig. 1a), the isotopic pattern (Fig. 1b) of the pseudomolecular ion *m/z* 163.06009 was registered, and the distribution of which could be linked to $C_6H_{11}O_5^+$ with a theoretical *m/z* equal to 163.06010. The mass spectra of the molecular ion and the secondary fragment ions for D-3-deoxyglucosone were presented in Fig. 1a,c.

During the positive ion collection mode, the protonated target metabolite was instable and the C–C bond of which was easy to be destroyed and then produced more stable characteristic fragment ions including $C_2H_5O_2^+$ (*m/z* 61.02841) and $C_3H_5O_2^+$ (*m/z* 73.02814) by McLafferty rearrangement reactions (rH_B and rH_C). In addition, after protonation, D-3-deoxyglucose was also easily cleaved into characteristic fragments with a three-stage positive carbon ion stable structure i.e. $C_6H_8O_4^{\bullet+}$ (*m/z* 144.04171), $C_5H_{10}O_4^{\bullet+}$ (*m/z* 134.05736) and $C_4H_7O_3^+$ (*m/z* 103.03897), by removing a water molecule or continuous i-fracture and $rH_{1,2}$ rearrangement reaction ($rH_{1,2}$) (Fig. 1d). Through applying MS-IOP analysis strategy, a total of 706 metabolites were identified from screened candidates in positive and negative ion collection modes of mass spectrometry.

2.6. Method validation for metabolomic

The detecting method was following the European Union SANTE/12,682/2019 guidelines and the methodological parameters including linearity, limit of quantitation (LOQ), accuracy, matrix effect and precision were investigated in this study. The LOQs were calculated by diluting the standards until the ratio of signals to noises values equal to 10. Accuracy and precision were assessed based on 3 spiked concentrations of metabolites (LOQ, 2 × LOQ and 4 × LOQ) in sextuplicate. Precision experiments of intraday and interday were performed to verify the precision of the method and expressed as the coefficient of variation of six technical repetitions of each experiment. Linearity evaluation was conducted at different contents ranging from 0.1 $\mu g\ L^{-1}$ to 10000.0 $\mu g\ L^{-1}$ for all metabolites dissolved in solvent and matrix extract. Matrix can influence the Q-Orbitrap signal intensity of the target compounds, which occurs by ion enhancement or ion suppression of the response intensity of MS and has profound effects on assay precision and accuracy in quantitative analysis, resulting in a recovery of some analytes greater than 100% [13]. *The Pharmacopoeia of the People's Republic of China 2020* stipulated that the recovery of the established quantitative method should be between 80% and 115%, when the content of the substances to be measured is on the magnitude of 10 ppm. It is necessary to evaluate the degree of matrix effects so that proper procedures can be taken to compensate or reduce the effects. The calibration curves of solvent and matrix were drawn with the response peak area of metabolites and the mass concentration as

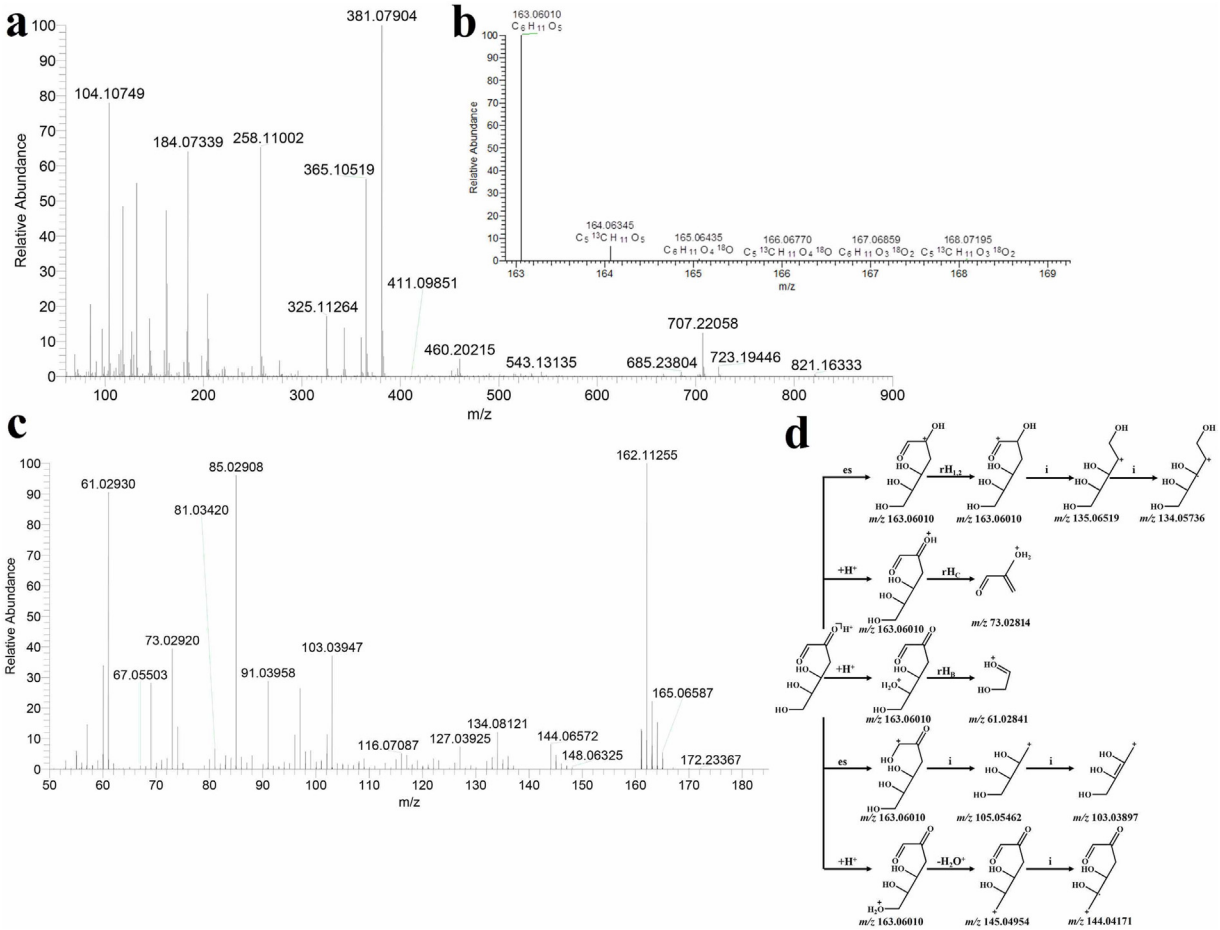

Fig. 1. Identification of metabolites by UHPLC/ESI Q-Orbitrap. (a) Full-MS spectrum of D-3-deoxyglucosone; (b) Isotopic pattern characteristic for D-3-deoxyglucosone; (c) Experimental MS/MS spectra of D-3-deoxyglucosone; (d) Fragmentation pathways of D-3-deoxyglucosone in UHPLC Q-Orbitrap.

the abscissa. The matrix effect was tested by comparing the slopes of the matrix-free calibration curves to the matrix-matched calibration curves. The matrix effect was investigated by the following formula:

$$\text{Matrix effect } (\%) = \left(\frac{S_{matrix}}{S_{solvent}} - 1\right) * 100 \quad (1)$$

where S_{matrix} is the slope of the calibration curve in the matrix and $S_{solvent}$ is the slope of the calibration curve in the solvent.

2.7. Protein identification and quantification

MS data were searched against *Streptococcus + thermophilus*. Fasta and *Lactobacillus + bulgaricus*. Fasta (33,004 and 18,575 total entries, respectively, downloaded 17 April 2022), and the precursor mass window of the initial search was set as 6 ppm.

The qualitative search for the proteomics followed the trypsin/P digestion rule with up to 2 missed cleavages sites allowed and mass tolerance of the fragment ion was set as 20 ppm. During the process of database search, the methylation of cysteine was defined as fixed modification, whereas the acetylation of protein N-terminal and the oxidation of methionine were defined as variable modification. The overall false discovery rate (FDR) for protein identification was set as 1% and the label-free quantification (LFQ) was performed for the proteins monitoring during the fermentation of brown fermented milk.

2.8. Analysis of chemometric and bioinformatics

The resulting dataset of qualitative and quantitative of substances was imported into SPSS 22.0, SIMCA and MetaboAnalyst 5.0 for further multivariate statistical analysis in *.CSV file format. As a dimensionality reduction method, unsupervised principal component analysis (PCA) was applied to visualize the intra group repeatability and inter-group differences, obtain an overview of correlation among determined metabolites or differentially deregulated proteins, evaluate the repeatability of data of metabolomics and proteomics and find possible outliers. Student's *t*-test was used for univariate analysis of the two sets of data to determine the significance of each metabolite [14]. The raw *p*-value of each metabolite in all cross-comparisons was revised by the Benjamini and Hochberg approach, and the adjusted *p*-value was obtained according to the following formulas [15].

$$FDR = p \times m/n \quad (2)$$

$$\text{adjusted } p\text{–value} = min\left(FDR_{(m)}, FDR_{(m+1)}\right) \quad (3)$$

where m is the maximum number of inspections to minimize FDR, p is the raw p value of m inspections, and n is the total number of inspections.

The variable importance in projection (VIP) score after mean-centering and Pareto scaling was applied to assess the importance of each variable in PLS-DA model and the statistical significance between different experimental conditions was evaluated by One-way analysis of variance (One-way ANOVA) followed by the Benjamini and Hochberg method and substances with adjusted p-value < 0.05 and VIP > 1 were defined as significantly different compounds. Pearson correlation analysis was carried out to further screen the substances with strong correlation ($|r| > 0.7$, adjusted p-value < 0.05) with the metabolism of HMRPs in brown fermented milk. In order to profile the microbial enzymes related to the inhibited formation and degradation of harmful products of

Maillard reaction, Gene Ontology (GO) Resource, Kyoto Encyclopedia of Genes and Genomes (KEGG) database and Carbohydrate-active enzymes (CAZymes) data bank were carried out for biological properties annotation of the identified proteins with significant differences between the experimental groups.

2.9. Sensory evaluation

Brown fermented milk samples with or without 2.5% pectin were evaluated by 30 panellists (15 men and 15 women) aged 20–28 years who were confirmed to have normal taste sensitivity and no reports of allergies. They were also familiar with the assessment of brown fermented milk and met the requirement of ISO 8568 (2012). The analyses were performed in sextuplicate with identical conditions, and five parameters according to ISO 22,935–2 (2009), including taste, aroma, texture, appearance and overall acceptability (Table S1), were evaluated based on a 6-point scale, with "0=strongly disliked" and "6=strongly liked". A two-month training consisted of five sessions were conducted according to the recommendations in ISO 8586–1 (1993) in sensory booths, under the same evaluation conditions as the formal samples to focus on sensory characteristics, refine terms and decrease group standard deviation. During the first session, after being familiar with the test procedure, panelists were presented with two samples of brown fermented milk with or without 2.5% pectin (each group was in triplicate) and requested to list the terms appropriate to describe the taste, aroma, texture, appearance of the samples. In this part, a total of 34 terms had been collected. The following four sessions were used to: *i* expose panellists to more yogurt samples and possibly identify new terms; *ii* reduce the total number of terms by eliminating redundant ones or those for which the panel could not reach a consensus; *iii* agree on precise definitions of the terms and on the tasting protocol; and *iv* practice the use of the rating scale and make sure that panellists rated samples coherently (Fig. 2). A total of 19 clearly defined terms (Table S1) were clarified.

Each sample was randomly coded by two digits and presented to the tasters. Twenty milliliter samples together with warm water as palate cleanser were poured into a glass cup and presented in a coded form. The sensory properties of brown fermented milk samples were recorded 60 s after swallowing and drinking 20 °C water to clean the taste buds before the next sample test. All the evaluators were in a normal physiological state and did not eat or smoke within 1 hour before evaluation, but they were permitted to drink water.

Sensory analysis was carried out in standard sensory evaluation laboratory ISO 8589 (2007), being equipped with white light source, controlled air flow and room temperature at 20–25 °C. The process was repeated three times for each sample and a 10-minute interval was maintained between each evaluation step. Final processed data were an average of the scores from all panelists and the Benjamini and Hochberg corrective method was used to correct obtained data of sensory evaluation.

2.10. Fuzzy mathematical synthesis analysis of sensory evaluation results

Voting number of sensory evaluation factors was converted into scores in evaluation domain and carried out by fuzzy mathematical synthesis analysis. The *p* value with multiple testing corrections was used to assess the significance of differences between samples.

Brown fermented milk with or without 2.5% pectin was used as the evaluation object set, i.e. $U = \{u_1, \ldots u_i, \ldots, u_n\}$ and taste, texture, odor, appearance and overall acceptance were taken as factor set, i.e. $X = \{x_1, x_2, x_3, x_4, x_5\}$ with equal evaluation weight coefficients $W = \{w_1, w_2, w_3, w_4, w_5\} = \{0.2, 0.2, 0.2, 0.2, 0.2\}$. The comment set $Y = \{y_1, y_2, y_3, y_4, y_5, y_6\}$ is composed of prefer (6), enjoy (5), like (4), general (3), dislike (2) and loathe (1).

After the panellists evaluated each factor of each sample the quotient of the number of evaluators at each level and the total number of evaluators under each evaluation factor of each group of samples were calculated and a fuzzy matrix was obtained,

$$A = [\alpha_1, \ldots, \alpha_i, \ldots, \alpha_6] \tag{4}$$

Establishing the fuzzy mathematics evaluation model,

$$Bi = W \times Ai \ (i = 1, 2, 3, 4, 5, 6) \tag{5}$$

The comprehensive evaluation of brown fermented milk is divided into the following:

$$Ti = Bi \times Y (i = 1, 2, 3, 4, 5, 6) \tag{6}$$

Comprehensive evaluations of sensory quality were calculated through a weighted average algorithm as shown in Table S2, and a high score indicated a good comprehensive sensory index.

3. Results and discussion

3.1. Overview of metabolites in brown fermented milk with different pectin additions

Univariate data analysis was firstly performed for the search of significantly different risk substances between conventional fermented milk and brown fermented milk groups (Fig. 3a). The volcano plot (Table S3), drawn according to the fold change (FC) of metabolites in the brown fermented milk (BM) and conventional fermented milk (CM) (BM/CM), indicated that methylglyoxal (ranged from 0.075 mg L^{-1} to 0.156 mg L^{-1}, FC 2.07, adjusted *p*-value = 0.019), 5-hydroxymethylfurfural (ranged from 0.0102 mg L^{-1} to 0.0267 mg L^{-1}, FC 2.61, adjusted *p*-value = 0.026) and acrylamide (ranged from 0.37 μg L^{-1} to 0.79 μg L^{-1}, FC 2.14, adjusted *p*-value = 0.041) were the HMRPs with significant differences (Fig. 3b).

During the heating process of dairy products, lactose condensed with protein to form lactose lysine, which can be further dehydrated and undergo Amadori rearrangement to form N-alkyl lactose. In addition, the rearrangement products can also form carbonyl compounds such as 5-hydroxymethylfurfural, acrylamide, and reduce ketones through enylation, deamination, and dehydration at pH 4–7. Due to the active chemical properties of reducing ketones, they can be decomposed into acetone aldehyde and acetic acid by Strecker degradation reaction [16]. The qualitative analysis was carried out based on selected secondary characteristic fragment ions, exact mass isotope distribution and RT, and the quantitative analysis was performed through a matrix-matched calibration curve external standard according to the following formula based on the peak area of the metabolite and its corresponding standard:

$$X_i = \frac{(c_i - c_{0i}) \times V}{m} \tag{7}$$

where X_i is the content of the target metabolite in the sample, c_i is the content of the target metabolite obtained by matrix-matched calibration curves, c_{0i} is the content of the standard added, V is the constant volume of the sample solution and m is the mass of the sample represented by the sample solution.

The quantitative results indicated that the addition of pectin could significantly reduce the content of methylglyoxal (ranged from 0.156 mg L^{-1} to 0.070 mg L^{-1}, adjusted *p*-value =1.2E-18) and acrylamide (ranged from 0.79 μg L^{-1} to 0.32 μg L^{-1}, adjusted *p*-value =1.0E-21) in brown fermented milk (Fig. 3c,d), and when the pectin concentration reached 2.5%, the concentration of HMRPs could be reduced to a level similar to that of conventional fermented milk (0.0075 mg L^{-1} for methylglyoxal, 0.0102 mg L^{-1} for 5-hydroxymethylfurfural and 0.37 μg L^{-1} for acrylamide) except 5-hydroxymethylfurfural (Fig. 3e) (ranged from 0.0267 to 0.0246 mg L^{-1}, adjusted *p*-value = 2.6E-4).

Pectin is considered as a polymer composed of three structures, including homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). HG is composed of R-(1,4)-linked D-galacturonic acid residues, which can be esterified at C-6 and O-acetylated at O-2 and O-3. The backbone of RGI is a repeating unit

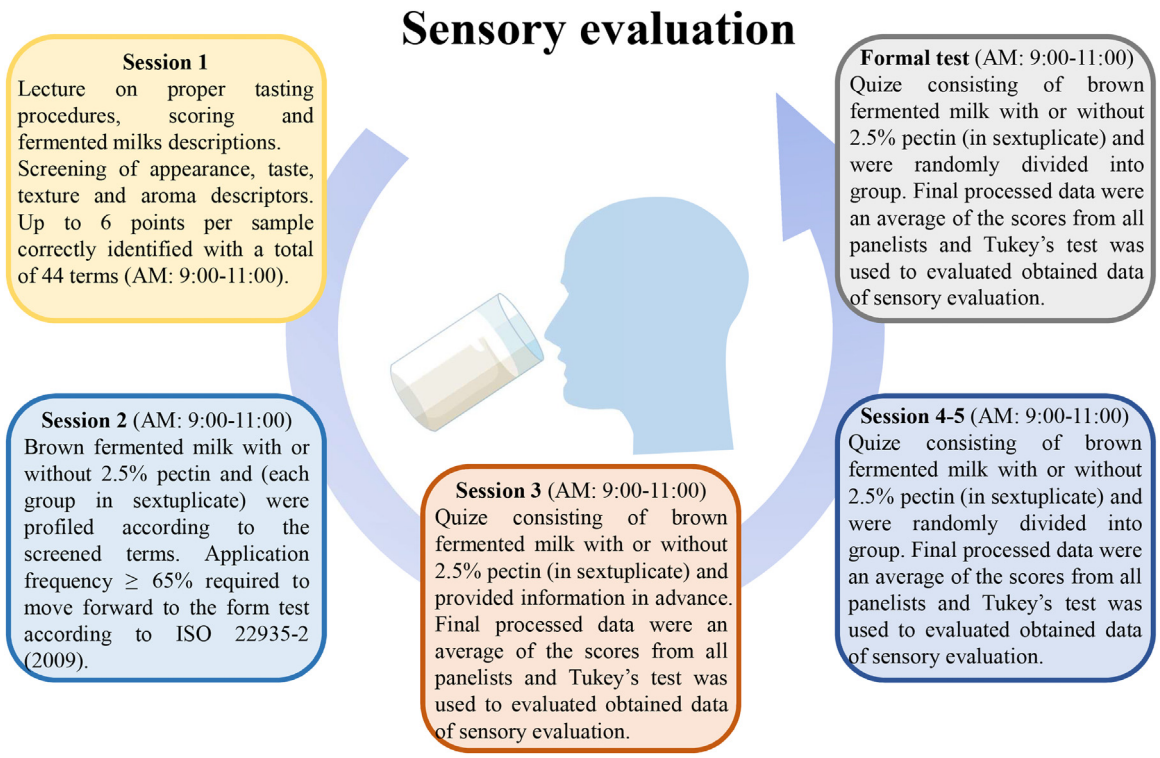

Fig. 2. Flowchart representation of the progression of training and test for panelists used in this study. A two-month training consisted of five sessions was conducted according to the recommendations in ISO 8586–1 (1993) in sensory booths, under the same evaluation conditions as the formal samples to focus on sensory characteristics, refine terms and decrease group standard deviation. During the first session, after being familiar with the test procedure, panelists were presented with two samples of brown fermented milk with or without 2.5% pectin (each group was in triplicate) and requested to list the terms appropriate to describe the taste, aroma, texture, appearance of the samples. The following four sessions were used to (i) expose panelists to more yogurt samples, and possibly identify new terms; (ii) reduce the total number of terms by eliminating redundant ones or those for which the panel could not reach a consensus; (iii) agree on precise definitions of the terms and on the tasting protocol; and (iv) practice the use of the rating scale and make sure that panelists rated samples coherently.

composed of dimer R-(1,2)-L-rhamnose-R-(1,4)-D-galacturonic acid, in which rhamnosyl residues can be substituted by neutral sugar side chains composed of galactosyl and arabinose residues at O-4. The primary chain of RG-II is polygalacturonic acid, and the side chain contains four kinds of oligosaccharides [17]. All these structural polymers are highly sensitively to enzymatic hydrolysis, acid hydrolysis and hydrothermal treatment (Fig. 3f). The hydrolysis of pectin from orange peel by the hydrothermal method showed that the hydrolysis of pectin was temperature dependent, and galactooligosaccharide was the main component of its hydrolysate [18]. Mada et al. revealed that the usage of pectin in yogurt could increase the microbial load [19]. During the browning process, pectin was hydrolysed to a certain extent into prebiotics that can be utilized by *Lactobacillus bulgari* and *Streptococcus thermophilus*, thus the amount of pectin added in brown fermented milk was higher than the literature value.

Quantitative results of metabolites showed that compared with conventional brown fermented milk, the content of intermediate metabolites, including glycerate-3P (Fig. 4a) (ranged from 0.0439 mg L^{-1} to 0.0128 mg L^{-1}, adjusted p-value = 1.5E-13 < 0.05) and D-glucose-6P (Fig. 4b), decreased significantly (ranged from 0.0726 mg L^{-1} to 0.0467 mg L^{-1}, adjusted p-value = 4.3E-14 < 0.05), while the downstream metabolites pyruvate (Fig. 4c) (ranged from 0.0024 mg L^{-1} to 0.0084 mg L^{-1}, adjusted p-value = 1.2E-11 < 0.05) and L-lactate (Fig. 4d) (ranged from 0.0057 mg L^{-1} to 0.0144 mg L^{-1}, adjusted p-value = 7.6E-11 < 0.05) were increased in brown fermented milk supplemented with pectin. In addition, the concentrations of galactose (Fig. 4e) (ranged from 0.0712 mg L^{-1} to 0.0893 mg L^{-1}, adjusted p-value = 3.4E-15 < 0.05) and D-glucose-1-phosphate (Fig. 4f) (ranged from 0.0692 mg L^{-1} to 0.0986 mg L^{-1}, adjusted p-value = 8.2E-15 < 0.05), the landmark metabolites of Leloir pathway, also showed an upward trend. These results demonstrated that in the brown fermented milk with pectin, lactic acid bacteria conduct energy metabolism through both Leloir pathway and glycolysis pathway. Previous studies have reported that lactic acid bacteria metabolized galactose oligosaccharides in following two ways: the transport of lactose permease (LacS) and the hydrolysis of galactosidase. Specifically, galactose oligosaccharide was transported by LacS and converted into glucose and galactose by β-galactosidase. Metabolites then entered the Leloir pathway and glycolysis, respectively. The other was translocation and phosphorylation by phosphotransferase (LacEF) and hydrolyzed by phosphogalactosidase. With the phosphorylation of galactooligosaccharides, phosphoenolpyruvate was converted to pyruvate and generates galactose-6-phosphate, which was subsequently hydrolysed by 6-phospho-beta-galactosidase (LacG) and converted to glucose and galactose and then entered the Leloir pathway and glycolytic metabolism [20]. Galactose-6-phosphate (Fig. 4g) (ranged from 0.0856 mg L^{-1} to 0.1175 mg L^{-1}, adjusted p-value = 2.0E-16 < 0.05) and phosphoenolpyruvate (Fig. 4h) (ranged from 0.0106 mg L^{-1} to 0.0396 mg L^{-1}, adjusted p-value = 4.7E-15 < 0.05), signature metabolites of LacEF/LacG, were increased significantly, demonstrating that the LacEF/LacG metabolic pathway was the main pathway of galactooligosaccharide metabolism in this study (Fig. 4i).

In addition, the evidence proved that LacEF-dependent utilization of lactose only appears in *Lactococcus lactis subsp, Lactis, Streptococcus mutans* and *Lactobacillus zeae* [21]. It can be speculated that the addition of pectin may affect the energy metabolism of *Streptococcus thermophilus*, while the energy metabolism of *L. bulgaricus* is hardly affected.

The correlation coefficients (R^2) of all the metabolites were higher than 0.99 (adjusted p-value = 1.4E-3 < 0.05) at the concentration levels of 0.2–10000.0 $\mu g\ L^{-1}$ and the LOQs of the established method was

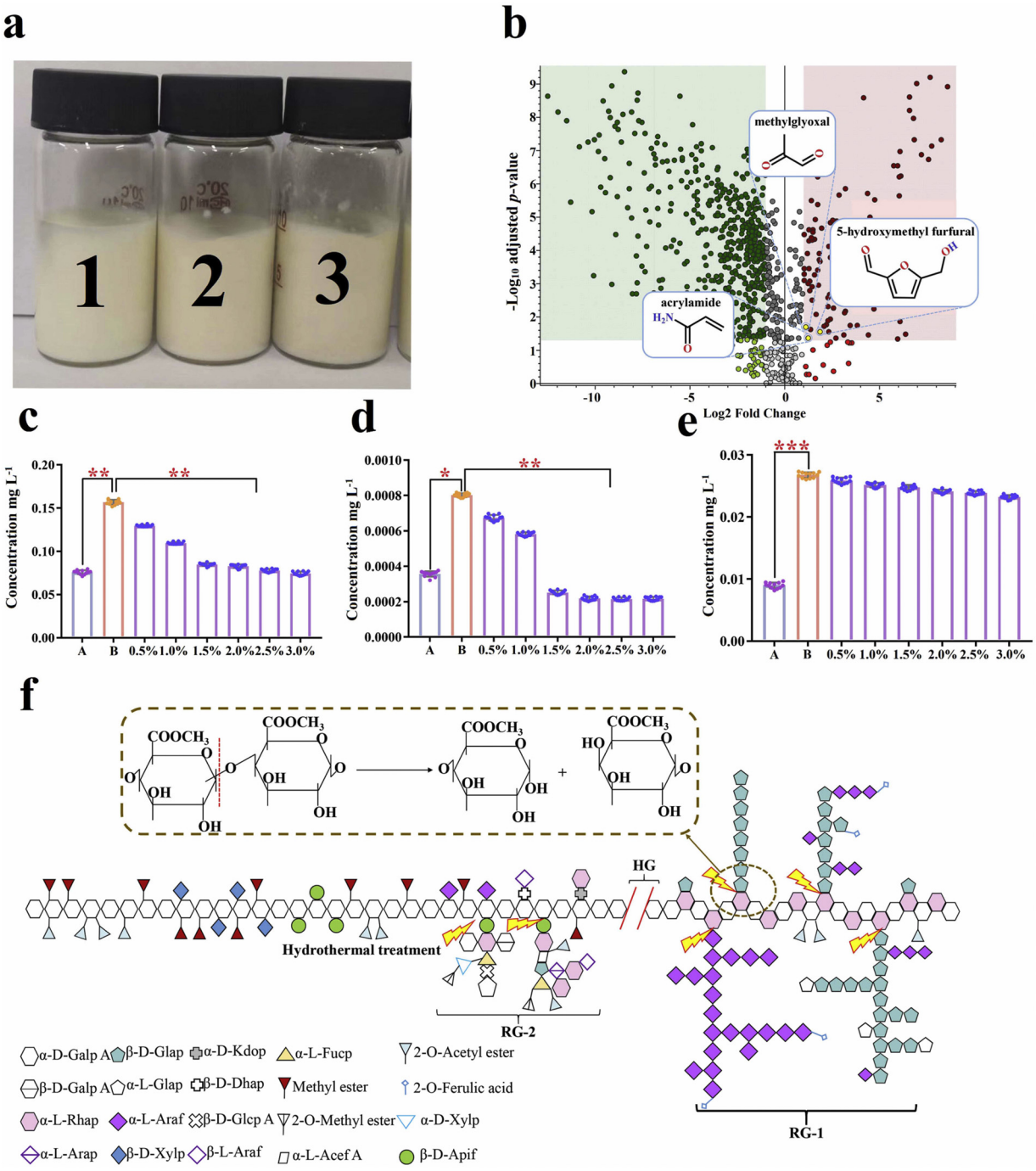

Fig. 3. Quantitative metabolomics analysis of conventional fermented milk, brown fermented milk (labeled A) and brown fermented milk (labeled B) with different concentrations of pectin (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%). (a) Conventional fermented milk 1, brown fermented milk 2 and brown fermented milk with addition of 2.5% pectin 3; (b) Volcano plots of the significantly differential metabolites (brown fermented milk with addition of 2.5% pectin/brown fermented milk). Two-sided Wilcoxon rank-sum tests followed by the Benjamini-Hochberg (BH) multiple comparison test with adjusted *p*-value ⟨ 0.05 and fold change ⟩ 2 or < 0.5. Metabolites significantly increased or decreased were colored in red and green, respectively; (c), (d), and (e) Quantitative results of methylglyoxal, acrylamide and 5-hydroxymethylfural, respectively (n = 24). Data represented the mean ± s.e.m. (One-way ANOVA test followed by the Benjamini and Hochberg corrective method, adjusted *p*-value < 0.001: ***; 0.001 ≤ adjusted *p*-value < 0.01: **; 0.01 ≤ adjusted *p*-value < 0.05: *; (f) The high temperature during the browning process of brown fermented milk and the acidic environment caused by metabolism of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* resulted in the breaking of α−1,4-D-glycosidic bond in pectin polygalacturonic acid chain and the generation of single or oligogalacturonic acids. The structure of pectin is referenced in reference [28] with permission from Elsevier.

ranged from 0.1 to 15.0 $\mu g\ L^{-1}$, which indicated that the method had a good sensitivity. The relative recovery was utilized to measure the accuracy of the method and the results of which were ranged between 89.1% and 112.5%. Relative standard deviations (RSDs) of intra-day and inter-day precision experiments were 1.3%–8.4% and 2.1%–9.4%, respectively, indicating that the method was accurate [22].

3.2. Profiling of substances related to the metabolism of HMRPs

Metabolite profiling was coupled with stoichiometry to direct the production strategy of brown fermented milk, with the goal of reducing the production of HMRPs and clarify the mechanism of pectin inhibiting

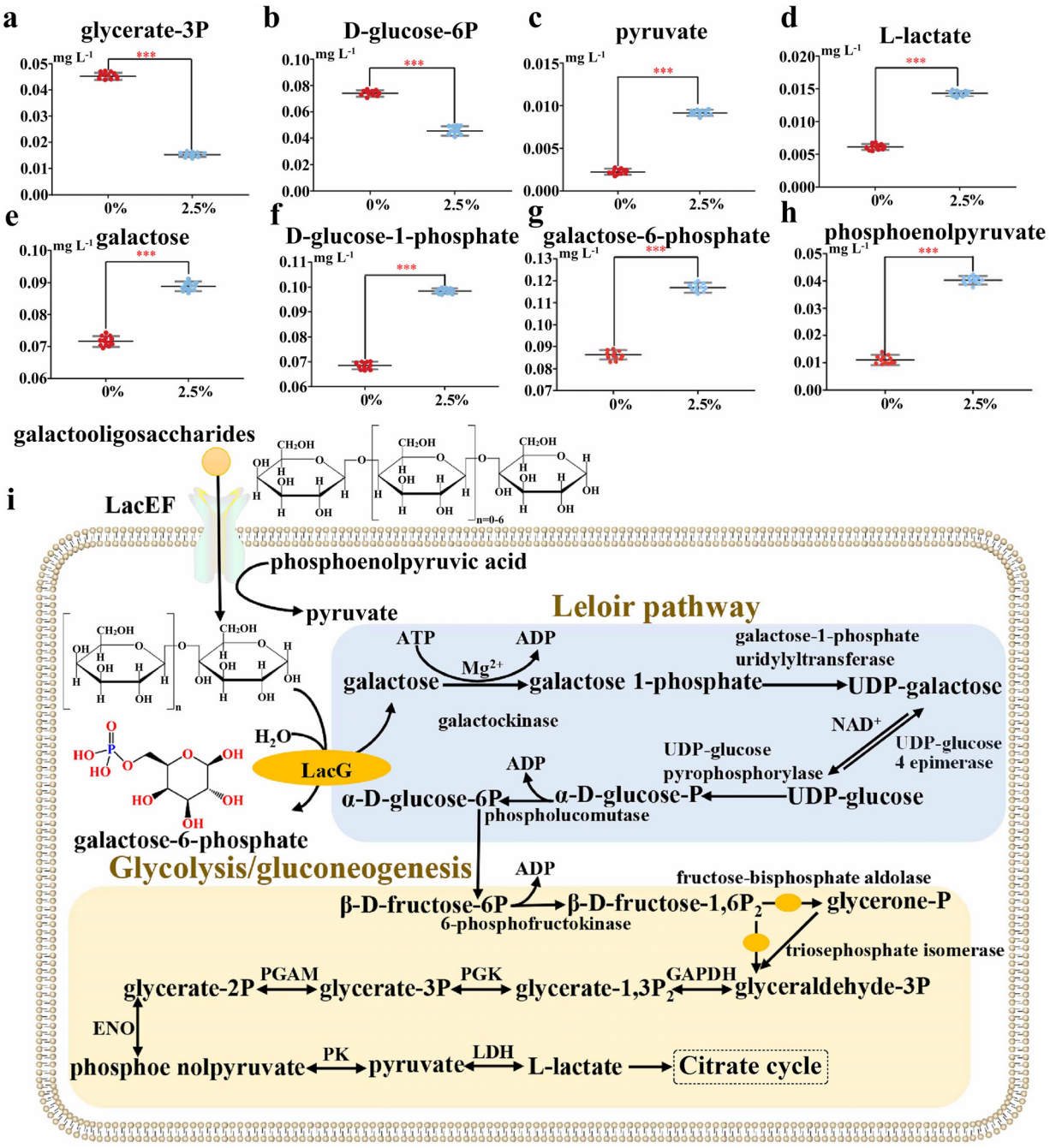

Fig. 4. Quantitative metabolomics analysis of brown fermented milk with addition of 2.5% pectin. From (a) to (h) Quantitative results of glycerate-3P, D-glucose-6P, pyruvate, L-lactate, galactose, D-glucose-1-phosphate, galactose-6-phosphate and phosphoenolpuryvate, respectively (n = 24). Data represented the mean ± s.e.m. and Student's t-test was used. (adjusted p-value < 0.001: ***; 0.001 ≤ adjusted p-value < 0.01: **; 0.01 ≤ adjusted p-value < 0.05: *); (i) Under the action of LacEF, galactose oligomer enters the cell of Streptococcus thermophilus and phosphorylates to galactose-6-phosphate, which can be further hydrolyzed by LacG to glucose and galactose and enter the Leloir pathway and glycolytic metabolism to provide energy for the life activities of *Streptococcus thermophilus*. This is the first report of the metabolic pathway of galactose oligosaccharide in *Streptococcus thermophilus*.

the production of harmful products. The difference in metabolite abundance in eight pectin addition experimental groups is depicted in Fig. 5a. As shown in the score plot, the QC samples were plotted near the center of the PCA and presented well gathered, demonstrating a good repeatability and reliability of the developed method during LC-MS analysis of all samples. The components revealed a significant separation among 7 fermented milk samples, and hierarchical cluster analysis showed that the milk samples with pectin addition of 2.5% and 3.0% were the closest to the components of conventional fermented milk, which was consistent with the quantitative results of HMRPs (Fig. 5b). PLS-DA analysis was utilized for the discriminant analysis of brown fermented milk samples and the important variables for differentiation between groups with various amounts of pectin were observed using VIP-value and p value with multiple testing corrections.

Value of the interpretation energy (R2X) and prediction ability (Q2) of the supervised partial least squares discriminant analysis were 0.968 and 0.931 (Fig. 5c), respectively, and the 200-permutation test confirmed the validity of the PLS-DA model established in this research

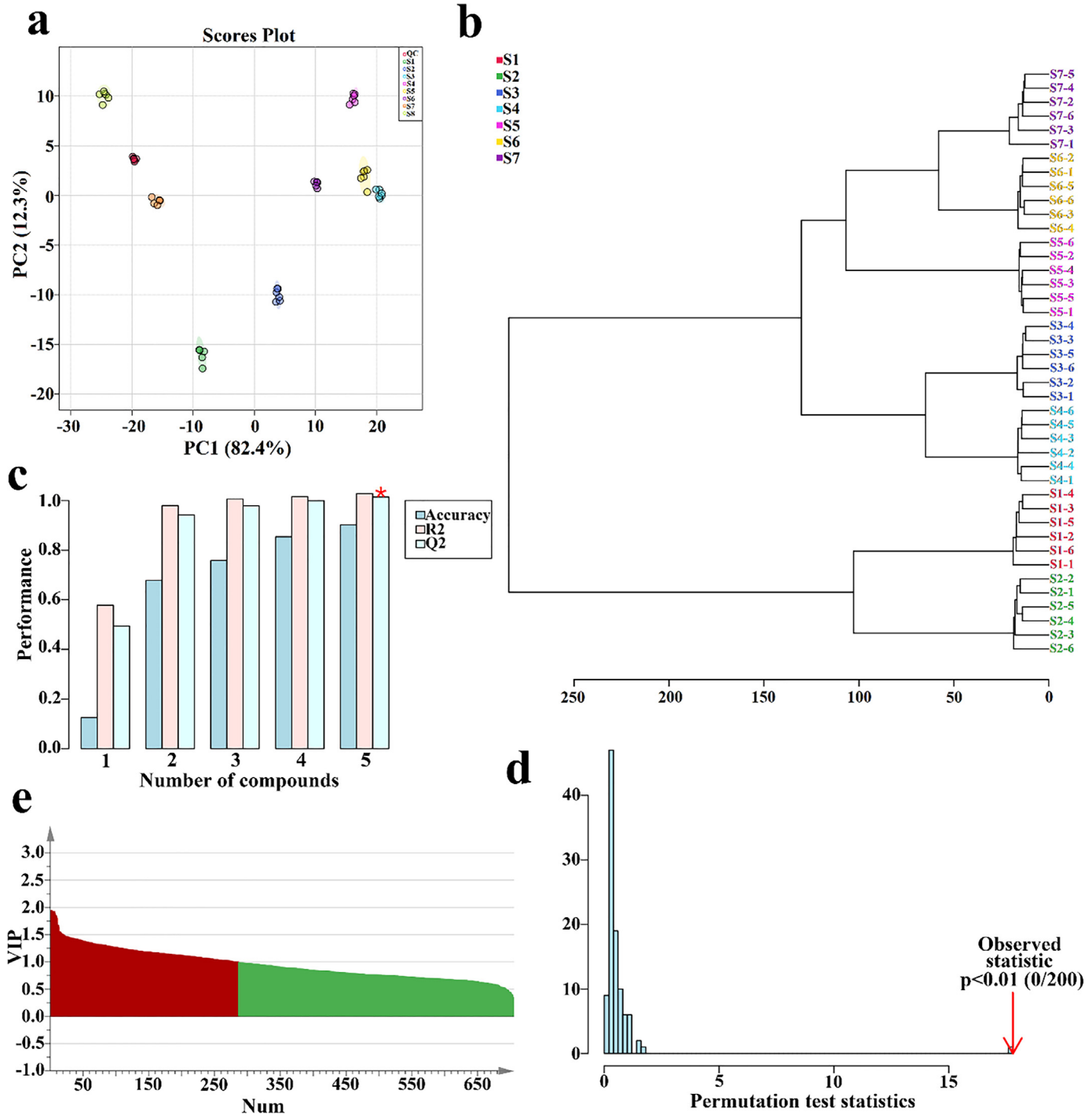

Fig. 5. Chemometric analysis of conventional fermented milk, brown fermented milk and brown fermented milk with different concentrations of pectin. (a) PCA plot distributed QC samples (n = 54), brown fermented milk with different concentrations of pectin (0.5% S1, 1% S2, 1.5% S3, 2% S4, 2.5% S5, 3% S6; n = 24), brown fermented milk S7 (n = 24) and conventional fermented milk S8 (n = 24) according to metabolites detected from untargeted metabolomics; (b) Hierarchical cluster analysis of conventional fermented milk (S7) and brown fermented milk with different concentrations of pectin (0.5% S1, 1% S2, 1.5% S3, 2% S4, 2.5% S5, 3% S6). Each group of samples was performed six replicates and the samples of each experiment were analyzed in quadruplicate as the technical repeats (n = 6 × 4); (c) Cross validation histogram of three performances (prediction accuracy, R2 and Q2) of PLS-DA; (d) Result of permutation testing for PLS-DA (200 times, adjusted p-value <0.05); (e) Results of VIP-value between brown fermented milk and brown fermented milk with different concentrations of pectin (0.5% S1, 1% S2, 1.5% S3, 2% S4, 2.5% S5, 3% S6) the red area is the distribution of substances with VIP > 1 and adjusted p-value < 0.05.

(p = 4.6E-68, adjusted p-value = 8.1E-66) (Fig. 5d). As depicted in Fig. 5e, a total of 198 substances (VIP ≥ 1, adjusted p-value < 0.05, red part in the plot) were considered to be significantly different metabolites and 51 compounds were identified as having a strong correlation with the generation of HMRPs (|r| > 0.7, adjusted p-value < 0.05) (Table S4). Among which, 39 variables displayed a strong negative correlation with Maillard reaction products, which relate to the degradation of HMRPs, while the remaining 12 substances showed the opposite behavior, which may be the factor to inhibit the formation of HMRPs (Fig. 6a). Hypergeometric distribution and topological analysis were applied for pathway

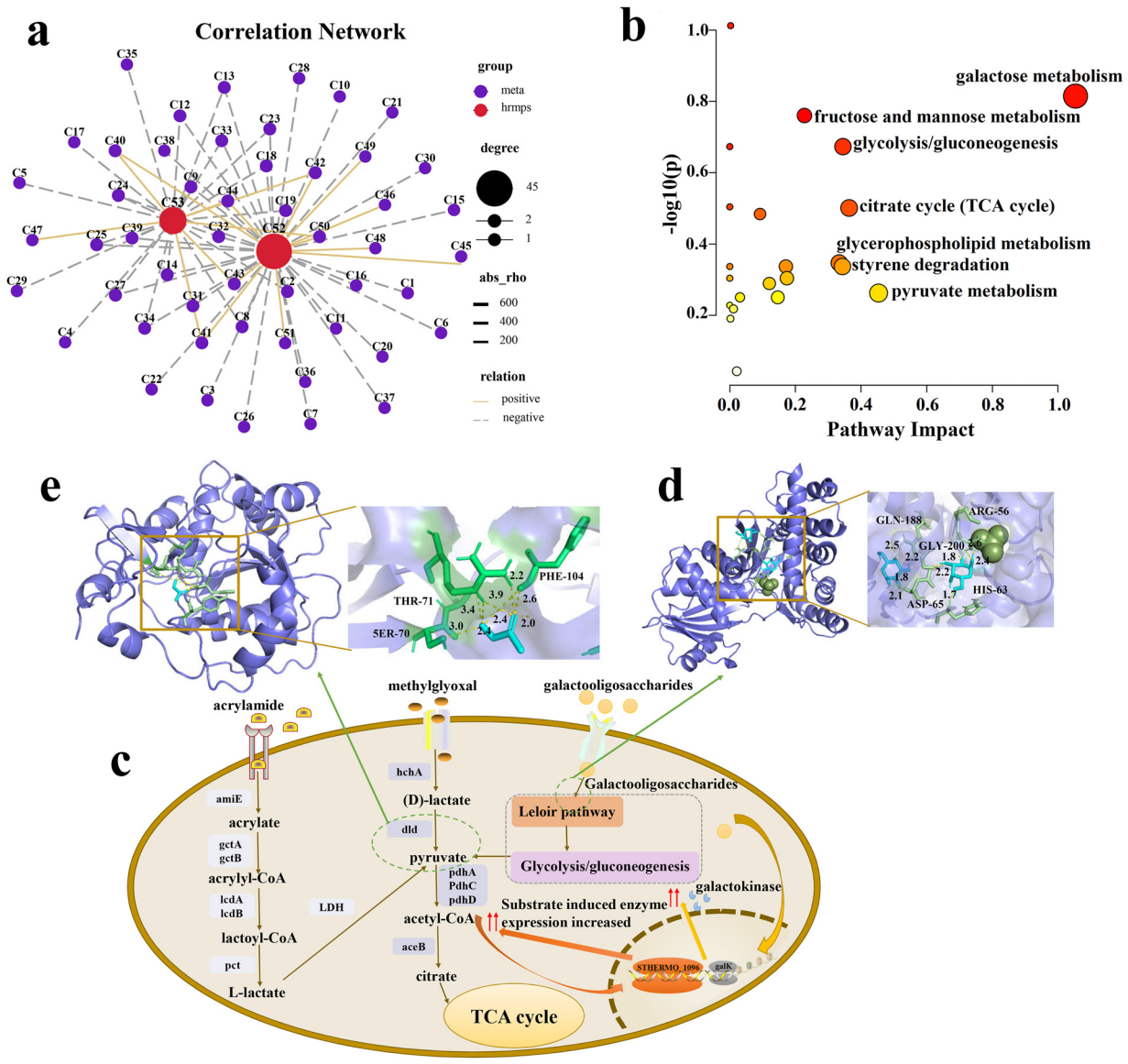

Fig. 6. Mechanism of pectin intervention on HMRPs degradation of brown fermented milk. (a) Correlation networks of acrylamide (C52), methylglyoxal (C53) and significant difference metabolites (VIP > 1, adjusted *p*-value < 0.05) related to the HMRPs degradation. Detailed data of correlation analysis were provided in Table S4. Nodes and edges are color-coded by Pearson's correlation coefficient. The gray dotted line represents negative correlation while the yellow solid line is the symbol of positive correlation. The width of the line represents the strength of correlation and the size of the dot represents the number of related objects; (b) Bubble plots of altered metabolic pathways in related to the HMRPs degradation (hypergeometric test). Bubble area is proportional to the impact of each pathway with color denoting the significance from highest (in red) to lowest (in white); (c) Mechanism of pectin accelerating degradation of acrylamide and methylglyoxal by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in brown fermented milk. Methylglyoxal is metabolized into pyruvate under the action of D-lactate dehydrogenase (hchA) and D-lactate dehydrogenase (DLD) expressed by *Lactobacillus bulgaricus* and further converted into citrate to participate in the tricarboxylic acid cycle (TCA cycle) under the catalysis of pyruvate dehydrogenase E1 component alpha subunit (pdhA), pyruvate dehydrogenase E2 component (pdhC), dihydrolipoamide dehydrogenase (pdhD) and malate synthase (aceB) expressed by *Streptococcus thermophilus*. Acrylamide can be converted into pyruvate and participate in the TCA cycle under the action of amidase and L-lactate dehydrogenase expressed by *Streptococcus thermophilus*. The addition of 2.5% pectin accelerated the flow of pyruvate to the TCA cycle (Citrate cycle), which effectively avoided the accumulation of methylglyoxal and acrylamide metabolites, maintained a high enzymatic catalytic efficiency, and accelerated the degradation of HMRPs. The mechanism of reducing acrylamide and methylglyoxal in brown fermented milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was clarified for the first time, which provided a novel insight for the control of HMRPs in brown fermented milk; (d) Molecular docking model of galactose (CAS 3646–73–9) and aldose 1-epimerase (PDB ID 1MN0); (e) Molecular docking model of pyruvate (CAS 57–60–3) and D-lactate dehydrogenase (PDB ID 1J49).

enrichment involved in differential metabolites and linked to biological significance. The abscissa pathway impact was calculated adding up the importance measures of each of the matched metabolites and then dividing by the sum of the importance measures of all metabolites in each pathway. The vertical coordinate $\log_{10}(p)$ value was preliminarily calculated from the pathway enrichment analysis (hypergeometric test) according to formula (8) and then corrected by the Benjamini and Hochberg approach to obtain the adjusted *p*-value according to formula (2) and (3) and then obtained by logarithmic conversion with 10 as the base ($\log_{10}(p)$). An adjusted *p*-value < 0.05 and impact value > 0.1 were identified as significantly related pathways under the study conditions.

$$\text{raw } p\text{–value} = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}} \tag{8}$$

Fig. 7. Quantitative proteomic analysis of enzymes related to HMRPs degradation. (a) Identification of peptides by UHPLC/ESI Q-Orbitrap; (b) Heatmap comparison of the enzymes related to the degradation of HMRPs (n = 24) according to the VIP-value based on PLS-DA model and One-way ANOVA test followed the Benjamini and Hochberg corrective method with adjusted p-value < 0.05. Z-scores were calculated using the average intensities of protein in samples; (c) Sankey Plot illustrated the names, genes, sources and metabolic pathways involved in the significantly down regulated enzymes related to the degradation of HMRPs, according to the VIP-value based on PLS-DA model and One-way ANOVA test followed the Benjamini and Hochberg corrective method with adjusted p-value < 0.05 (the Sankey diagram was built using SankeyMATIC online tool).

where N is the number of genes with pathway annotation in all genes; n is the number of differentially expressed genes in N; M is the number of genes annotated as a specific pathway among all genes and m is the number of differentially expressed genes annotated as a specific pathway [23].

Results of metabolic pathway analysis showed that a total of 22 pathways were involved in the metabolism of HMRPs (Fig. 6b). Fructose and mannose metabolism, galactose metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, glycerophospholipid metabolism, styrene degradation and citrate cycle (TCA cycle) were identified as the significant pathways. It followed that, acrylamide can be metabolized into L-lactate by styrene degradation and enters the tricarboxylic acid cycle through pyruvate metabolism, while methylglyoxal can also participate in the tricarboxylic acid cycle through pyruvate metabolism to provide energy for the life activities of lactic acid bacteria. Whereas, the pathway enrichment analysis indicated that 5-hydroxymethyl furfural was not included in any metabolic pathway, which indicated that the life activities of *L. bulgaricus* and *Streptococcus thermophilus* in brown fermented milk could not promote the metabolism of 5-hydroxymethylfurfural, which was consistent with the quantitative results (Fig. 6c).

3.3. Profiles of proteins in different pectin addition treatments

Proteomics based on a label-free quantitation analysis was subsequently carried out to identify and verify the enzymes responsible for metabolic regulation (Fig. 7a). A total of 571 peptides were identified, and the length of which was ranged from 7 to 43 amino acids, demonstrating that the mass spectrum information of peptides collected in this experiment was reasonable. The mass errors of peptides were distributed within 5 ppm, which indicated that the identification method was accurate. Andromeda scores of 88.47% peptides were higher than 60, and the median score was 95.57, which profiles the matching degree between the obtained peptide spectrum and the common theory and proved that the identified peptides were credible [24]. The variable importance for the projection obtained from the PLS-DA model was utilized to measure the influence intensity and interpretation abilities of the expression patterns of various proteins based on the classification and discrimination of each sample. The value of R2X is 0.957 and Q2 is 0.928. Results of permutation test (200 time) after multiple testing corrections indicated that the original model was not over fitted (adjusted p-value = 1.1E-76 < 0.05). All of the above results showed that the model had good predicative performance. These peptides were assembled into 49 proteins (Table S5), 25 of which were significantly upregulated and belonged to 18 classes of enzymes (VIP > 1, adjusted p-value < 0.05) in the pectin-added groups (Fig. 7b), while 2 proteins presented the opposite trends through partial least squares discriminant analysis.

D-lactate dehydratase (EC 4.2.1.130) and D-lactate dehydrogenase (EC 1.1.1.28) secreted by *L. bulgaricus* were the key enzymes in the metabolism of methylglyoxal to pyruvate. Enzymes originated from streptococcus thermophilus, including pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1), pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) (EC 2.3.1.12), dihydrolipoamide dehydrogenase (EC 1.8.1.4) and malate synthase (EC 2.3.3.9) were responsible for catalyzing conversion of pyruvate to (*S*)-malate, which was an intermediate metabolite of TCA cycle. Amidase (EC 3.5.1.4) identified based on Streptococcus thermophilus protein data was the rate limiting enzyme of acrylamide metabolism, which can metabolize acrylamide into acrylic acid and the metabolites can be

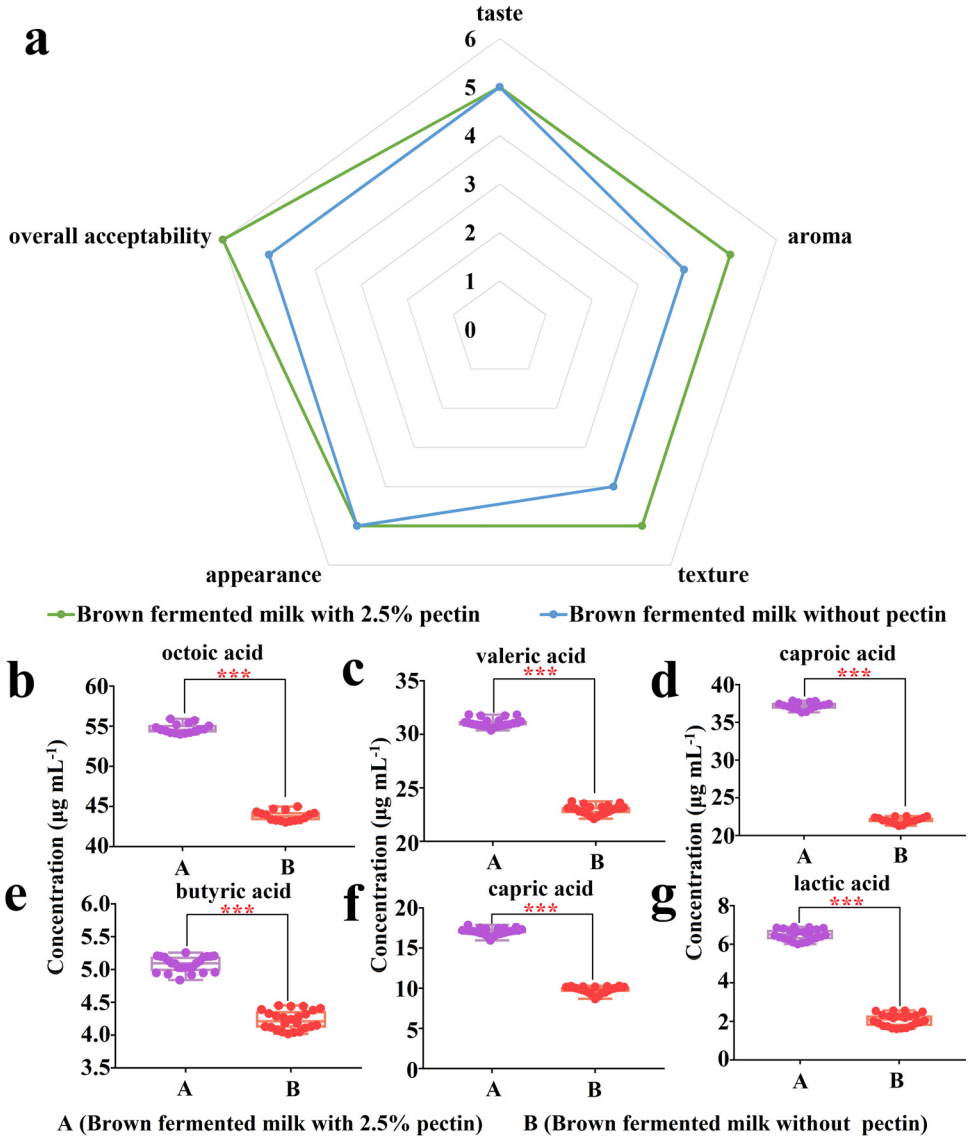

Fig. 8. Sensory evaluation and quantitative results of significant difference fatty acids of brown fermented milk and brown fermented milk with 2.5% pectin. (a) Radar chart of sensory evaluation results of brown fermented milk and brown fermented milk with 2.5% pectin through a 6-point scale (Benjamini and Hochberg corrective method); Quantitative results of significant fatty acids specifically include octoic acid (b), valeric acid (c), caproic acid (d), butyric acid (e), capric acid (f), lactic acid (g) in brown fermented milk with (A) or without (B) 2.5% pectin. Data represented the mean ± s.e.m. and Student's *t*-test was used. (adjusted p-value < 0.001: ***; 0.001 ≤ adjusted p-value < 0.01: **; 0.01 ≤ adjusted p-value < 0.05: *).

further catalyzed by L-lactate dehydrogenase (EC 1.1.1.27) secreted by *Streptococcus thermophilus* and participate in the metabolism of tricarboxylic acid cycle. Overall, these results indicated that the degradation of HRMs in brown fermented milk was the result of the joint action of *Streptococcus thermophilus* and *L. bulgaricus*.

In terms of the enzymes involved in energy metabolism, aldose 1-epimerase (EC 5.1.3.3), galactokinase (EC 2.7.1.6), hexose-1-phosphate uridylyl transferase (EC 2.7.7.12), glucose-1-phosphate uridylyl transferase (EC 2.7.7.9) and UDP-glucose 4-epimerase (EC 5.1.3.2) secreted by *Streptococcus thermophilus* were identified as the proteins significantly up-regulated in the Leloir pathway. Glyceraldehyde 3-phosphatedehydrogenase (EC 1.2.1.12), phosphoglycerate kinase (EC 2.7.2.3), phosphoglycerate mutase (EC 5.4.2.11), enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40) were enzymes involved in the glycolysis/gluconeogenesis pathway and significantly up-regulated in brown fermented milk added with pectin, which was consistent with the quantitative results of metabolomics. It is worth noting that the results of quantitative proteomics showed that there was nonsignificant variation in the expression of enzymes secreted by *L. bulgaricus* involved in Leloir pathway and glycolysis/gluconeogenesis pathway, which indicated that the addition of pectin can mainly promote the energy metabolism of *Streptococcus thermophilus*, while the energy metabolism of *L. bulgaricus* was almost unaffected, which was consistent with the results of quantitative metabolomics analysis. Previous studies have demonstrated that the low synthesis of galactokinase is an important reason for the low utilization of galactose by *L. bulgaricus*. In addition, it also had been proved that the reverse transposon transport competition of lactose galactose inhibits the enzyme activity of galactokinase of *L. bulgaricus* (Fig. 7c) [25]. NAD-aldehyde dehydrogenase (EC 1.2.1.3) and alanine dehydrogenase (EC 1.4.1.1) were the significantly downregulated proteases belonging to *Streptococcus thermophilus*. The reduction of NAD-aldehyde dehydrogenase (EC 1.2.1.3) would inhibit the conversion of acetate to acetaldehyde in the metabolic process of *Streptococcus thermophilus*, resulting in the accumulation and efflux of acetate. Due to the lack of pyruvate for-

mate lyase, *L. bulgaricus* was unable to synthesize purine and short-chain fatty acids for growth, and the increase in short-chain fatty acids (such as acetate) was contribute to the growth of *L. bulgaricus* [26]. The down-regulation of alanine dehydrogenase (EC 1.4.1.1) prevented the conversion of pyruvate to L-alanine and caused pyruvate flow to the citrate cycle, which provide sufficient energy for the life activities of *Streptococcus thermophilus*.

3.4. Enzymes involved in the metabolism of HMRPs

Quantitative results of proteomics indicated that compared with brown fermented milk, enzymes involved in pyruvate metabolism increased significantly in the brown fermented milk added with 2.5% pectin, while the expression of enzymes regulating the metabolism of methylglyoxal and acrylamide had no significant change, although the contents of HMRPs decreased according to results of metabolomics (Fig. 7b). These were related to the feedback regulation mechanism of enzymes [27]. Metabolites reversibly combined with the allosteric center to change the spatial structure of the enzyme and reduced the catalytic efficiency, while the enzyme activity recovered after metabolites separate from the allosteric center. The acceleration of pyruvate metabolism caused by pectin (Fig. 6d) prevented the accumulation of metabolites of methylglyoxal and acrylamide (Fig. 6e), thus maintaining a high enzyme catalytic rate and accelerating the degradation of HMRPs.

3.5. Sensory evaluation

As shown in Fig. 8, the results of sensory evaluation indicated that the brown fermented milk with pectin obtains higher scores than conventional brown fermented one. Bioinformatics analysis showed that compared with conventional fermented milk, the metabolic pathway of pyrimidine metabolism, glutathione metabolism, glycerolipid metabolism, alanine, aspartate and glutamate metabolism and purine metabolism in brown fermented milk with pectin was significantly up-regulated. Quantitative results of metabolomics showed that the contents of short chain fatty acids including octoic acid (55.6 μg mL^{-1}), valeric acid (31.7 μg mL^{-1}), caproic acid (37.6 μg mL^{-1}), butyric acid (5.1 μg mL^{-1}), capric acid (17.0 μg mL^{-1}) and lactic acid (6.7 μg mL^{-1}) in brown fermented milk with pectin were significantly higher (VIP > 1, adjusted *p*-value ranged from 2.3E-5 to 1.2E-27 < 0.05) than those in the conventional Brown fermentation group (ocotic acid (44.7 μg mL^{-1}, adjusted *p*-value =1.2E-27), valeric acid (23.6 μg mL^{-1}, adjusted *p*-value =7.2E-22), caproic acid (22.4 μg mL^{-1}, adjusted *p*-value =3.5E-25), butyric acid (4.2 μg mL^{-1}, adjusted *p*-value =1.9E-6), capric acid (9.6 μg mL^{-1}, adjusted *p*-value =9.4E-12) and lactic acid (2.3 μg mL^{-1}, adjusted *p*-value =2.3E-5)). Caproic acid had been evidenced to contribute to cheesy aroma and valeric acid and capric were responsible for the sweet flavor.

This was consistent with the results of sensory evaluation that the brown fermented milk with 2.5% pectin had stronger butter, milky aromas. Lactic acid, malic acid and citric acid were non-volatile organic acid, which can donate to the pleasant sour taste of brown fermented milk. In addition, previous studies proved that lactic acid and citric acid were negatively correlated with bitterness, which was conducive to reducing the bitter taste produced in the browning process to a certain extent. Typical fresh substances, uridine, guanosine and glutamic acid also increased significantly after the addition of pectin, thus endowing the brown fermented milk with good flavor and taste. In terms of texture, brown fermented milk with pectin presented thicker characteristic, which was related to the gel properties of pectin. The carboxylate group of pectin that had not been completely hydrolyzed can be converted into carboxylic acid group by the acid system of brown fermented milk, which weakened the repulsion and hydration degree between pectin molecules and is conducive to the formation of gel by intermolecular association, thus made the brown fermented milk system have viscous texture.

4. Conclusion

Brown fermented milk has gained popularity due to its unique flavor, while the Maillard browning reaction could lead to an increase in harmful products, which should be effectively controlled. In this study, 51 substances related to the degradation of HMRPs were screened out through quantitative untargeted metabolomics-based UHPLC-Q-Orbitrap, and pyruvate metabolism was identified as the vital pathway of HMRP degradation by bioinformatics analysis. Galactooligosaccharide was proven to be the main degradation product of pectin in brown fermented milk, and the LacEF/LacG pathway was the approach for galactooligosaccharide metabolism of *Streptococcus thermophilus* in brown fermented milk supplemented with pectin. Twenty-five significantly differentially expressed proteins of *L. bulgaricus* and *Streptococcus thermophilus* were identified as enzymes related to energy metabolism based on label-free proteomics, and the degradation of HMRPs was the result of the synergistic effect of *Streptococcus thermophilus* and *L. bulgaricus* based on a feedback regulation mechanism. Hydrolysate of pectin could be utilized as prebiotics by *Streptococcus thermophilus* and up-regulate expression of enzymes involved in energy metabolism, thus promoting acrylamide and methylglyoxal to enter TCA cycle. NAD-aldehyde dehydrogenase and alanine dehydrogenase were down-regulated enzymes belonging to *Streptococcus thermophilus* which is beneficial to efflux of acetate, promote proliferation of *L. bulgaricus*, prevent the conversion of pyruvate to L-alanine and lead to the flow of pyruvate to the TCA cycle for energy metabolism. Sensory evaluation revealed that addition of pectin endowed the brown fermented milk with better flavor and texture, which was related to the fact that pectin degradation up regulated the fatty acid metabolism of microorganism and accelerated the production of short chain fatty acid. This study provided an insight into the degradation mechanism of harmful products of Maillard reaction with the aim to lower the intake of dietary advanced glycation end products.

Declaration of competing interest

The authors declare that they have no conflicts of interest in this work.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fmre.2022.12.003.

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